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Direct assessment of symbiotically fixed nitrogen in the rhizosphere of alfalfa

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Abstract

Rhizodeposition has been proposed as one mechanism for the accumulation of significant amounts of N in soil during legume growth. The objective of this experiment was to directly quantify losses of symbiotically fixed N from living alfalfa (*Medicago sativa* L.) roots to the rhizosphere. We used ^{15}N-labeled N_2 gas to tag recently fixed N in three alfalfa lines [cv. Saranac, Ineffective Saranac (an ineffectively nodulated line), and an unnamed line in early stages of selection for apparent N excretion] growing in 1-m long polyvinylchloride drainage lysimeters in loamy sand soil in a greenhouse. Plants were in the late vegetative to flowering growth stage during the 2-day labelling period. We determined the fate of this fixed N in various plant organs and soil after a short equilibration period (2 to 4 days) and after one regrowth period (35 to 37 days). Extrapolated N_2 fixation rates (46 to 77 μg plant^{-1} h^{-1}) were similar to rates others have measured in the field. Although there was significant accretion of total N in rhizosphere compared to bulk soil, less than 1% was derived from newly fixed N and there were no differences between the 'excreting' line and Saranac. Loss of N in percolate water was small. These results provide the first direct evidence that little net loss of symbiotically-fixed N occurs from living alfalfa roots into surrounding soil. In addition, these results confirm our earlier findings, which depended on indirect ^{15}N labelling techniques. Net N accumulation in soil during alfalfa growth is likely due to other processes, such as decomposition of roots, nodules, and above ground litter, rather than to N excretion from living roots and nodules.

Introduction

Symbiotic N_2 fixation is a significant N source in agricultural ecosystems (Heichel, 1987). The widely grown forage legume, alfalfa (*Medicago sativa* L.), fixes about one million metric tons of N each year in eight USA Corn Belt states (Peterson and Russelle, 1991). This N input from only 8% of the cropland is large in comparison to the four million metric tons of fertilizer N that are applied annually to all other crops in the region.

Much of the fixed N in forage legumes is harvested and fed to animals, but evidence from a number of experiments using different methodologies indicates that alfalfa and other legumes can deposit significant amounts of N in the soil during growth. Both classical (Lyon and Bizzell, 1934) and contemporary (Andrén et al., 1990; Keeney, 1979) research estimated that net accre-

tions of 50 to 100 kg N ha^{-1} yr^{-1} occur in soil during alfalfa growth. The largest estimates of apparent N deposition by legumes is for pigeon pea [*Cajanus cajan* (L.) Huth], where symbiotically fixed N remaining in soil nearly equalled that in the shoot at maturity (Poth et al., 1986). Transfer of symbiotically fixed N from legumes to grasses growing in mixtures has been well documented, although such transfer is not always evident. For example, Burity et al. (1989) estimated that 20 kg N ha^{-1} was transferred from alfalfa to associated grasses during regrowth between two consecutive harvests, but Ledgard et al. (1985) did not detect N transfer from alfalfa to annual ryegrass (*Lolium rigidum* L.) over a 36-day field study. Amount of N transferred depends on distance between the legume and grass (Brophy et al., 1987), age of the plants (Ofosu-Budu et al., 1990), and environmental conditions (Ledgard, 1991; Wilson, 1940).

The major pathway(s) of N transfer must be determined to maximize internal recycling of symbiotically fixed N in grass-legume associations. For other management situations, minimizing losses of symbiotically fixed N from legumes may help reduce the potential for ground water contamination (Russelle and Hargrove, 1989).

Potential pathways of N loss from the legume include: excretion of N from roots and nodules (Lory et al., 1992; Ta et al., 1986); senescence and degradation of nodules (Vance et al., 1982) or roots (Butler et al., 1959; Jones, 1943); direct interconnection of grass and legume roots via mycorrhizal fungi (Haystead et al., 1988); ammonia loss from legume herbage and reabsorption by grass herbage (Lemon and Van Houtte, 1980); and movement of N from legume herbage to the soil via leaching or decomposition of surface litter.

Losses of nitrogenous substances from growing alfalfa appear to be small (1 to 4.5% of total symbiotically fixed N) under hydroponic (Ta et al., 1986), gnotobiotic (Brophy and Heichel, 1989), and soil (Lory et al., 1992) conditions. Lory et al. (1992) measured N deposition in the field indirectly with ^{15}N isotope dilution; there are no reports of N losses to soil measured directly. The objective of this experiment was to directly quantify the net amount of symbiotically fixed N in the rhizosphere of established alfalfa.

Materials and methods

Growth conditions

The Ap and B2/C1 horizons of a Hubbard loamy sand soil (Udorthentic Haploboroll) were collected separately at the Sand Plain Irrigation Research Farm near Becker, Minnesota, USA. Field-moist soil was screened (1.2-cm mesh) to remove rocks and larger plant residues, limed (1.9 g CaO kg^{-1} topsoil) and fertilized (3.0 g KCl kg^{-1} topsoil, other nutrients being adequate) according to Univ. of Minnesota recommendations (Rehm et al., 1985), and then packed to approx. 1.27 Mg m^{-3} dry bulk density into 15-cm diam. by 1-m long polyvinylchloride (PVC) tubes, with 30 cm of Ap horizon soil (topsoil) over 70 cm of B2/C1 soil (subsoil). The PVC tubes were closed on the bottom end with PVC caps, after adding a layer of glass wool as a filter. The caps had been tapped to allow collection of leachate. All drainage lysimeters were placed in a rectangular array (about 19 cm on center) in an insulated, air-conditioned (15 to 20 °C) enclosure with soil surfaces level with the top of the enclosure.

The experiment was conducted with three alfalfa germplasms: (1) a host-mediated ineffectively nodulated line of alfalfa (Ineffective Saranac; Barnes et al., 1990) as the control, primarily to account for nonsymbiotic N_2 fixation in the rhizosphere; (2) a widely grown cultivar, Saranac; and (3) and unnamed line of alfalfa selected for apparent N excretion. The 'excreting' line had been selected first in situ from plants growing with grasses that were visually N-sufficient and then by the response of oats (*Avena sativa* L.) growing near these first cycle selections in sand benches in the greenhouse (D.K. Barnes, 1988, personal communication).

Seeds of the three alfalfa germplasms were inoculated with a commercial mixture of *Rhizobium meliloti* (Nitragin, Milwaukee, Wisconsin, USA) and grown in sand benches for 10 weeks. During the first 6 weeks, plants received no fertilizer N, which allowed recognition and removal of effectively nodulated individuals within the Ineffective Saranac germplasm. Fertilizer N was added to all benches thereafter to improve growth of the ineffectively nodulated alfalfa. Plants were prepared for transplanting at

the prebud growth stage by trimming both the shoots and roots to approx. 10 cm length, removing most thin roots from the tap root, and dipping the root in a commercial mixture of *Rhizobium meliloti* strains suspended in deionized water. Four plants from a single germplasm were transplanted to each lysimeter (equivalent to 225 plants m^{-2} based on rooting volume) on 27 June 1988. Germplasms were assigned randomly to adjacent lysimeters in each of 12 replicate blocks; six replicates for each of two planned sampling times. A set of three lysimeters, each containing one of the three germplasms, was considered a replicate block. All subsequent procedures were conducted on the three germplasms concurrently.

Deionized water was added as necessary to avoid plant drought stress. Excess deionized water was added periodically during the following 6 months and leachate was collected by applying a −30 to −40 kPa suction to the drainage tube, 24 hours after adding the water. Leachate water volume was measured and inorganic N concentration was determined on a Wescan Ammonia Analyzer (previously sold by Wescan Instruments Inc., Santa Clara, California, USA). Nutrient solution without N was added every 2 weeks after the second harvest (see following paragraph) to alleviate potential nutrient stresses. To help maintain growth of Ineffective Saranac, the small mineralized N supply from this sand soil was supplemented by $(NH_4)_2SO_4$ additions every 2 weeks to all germplasms at the equivalent of 1.0 g m^{-2}. Therefore, within each of the succeeding regrowth periods, plants received 2 to 3 g m^{-2} supplemental inorganic N, an amount not likely to cause significant decline in symbiotic N_2 fixation. High pressure Na lighting supplemented sunlight and extended photoperiod to 14 hours. Air temperatures in the greenhouse fluctuated between approx. 17 and 27 °C after late September, with higher maxima and minima during summer.

Herbage was harvested about 7 cm above the soil surface on 16 August, 19 September, 24 October, and 28 November. During the latter three harvests, successive replicates were harvested every other day to establish the staggered intervals needed during labelling and sampling. Each of the succeeding procedures was conducted in the same sequence.

^{15}N labelling

Labelling with ^{15}N began for the first replicate on 2 January 1989. Bags made of Saran (polyvinylidene, 50 μm thick, Kenton Bag Co., Kenton, Ohio, USA) were sealed with MSDF compound (Press-in-Place® caulk, 3M Co., St. Paul, Minnesota, USA) to the tops of each lysimeter in the replicate after connecting a gas circulating system that joined the treatments in parallel (Fig. 1). Saran is only slightly permeable to N_2 (Van Kessel, 1983). The system was tested for leaks, some of the gas was removed, and a mixture of $^{15}N_2$-Ar-O_2 was added to achieve an average ^{15}N concentration of about 5 atom percent in N_2, Ar, and O_2 concentrations of about 60%, 18%, and 21%, (vol/vol), respectively. The three lysimeters were exposed to this gas mixture for 48 h, with intermittant circulation to promote mixing, and gas samples were taken periodically for ^{15}N and CO_2 analysis. Gas

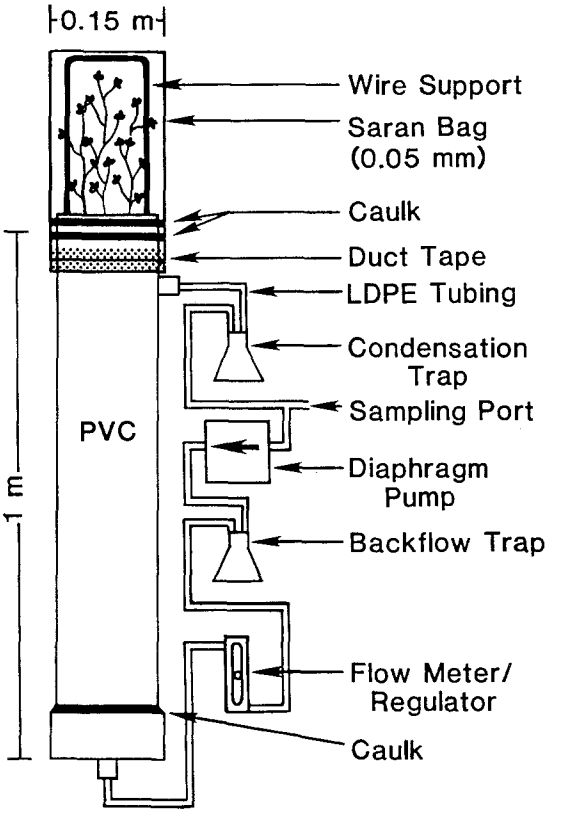

Fig. 1. Schematic diagram of gas circulating system used to label alfalfa with $^{15}N_2$. Three drainage lysimeters were connected in parallel with one pump and condensation trap system, and flow rate was adjusted with individual regulators.

samples were analyzed on a modified CEC21-620A isotope ratio mass spectrometer, using both the mass 29/28 and 30/28 ratios to determine ^{15}N abundance (Hauck, 1982). Samples were analyzed for CO_2 by standard infra-red absorption. Air temperature was monitored with a shaded thermocouple in each bag and in the external greenhouse atmosphere (Fig. 2). Plants were in the late vegetative to flowering stage of growth during labelling.

Equilibration of $^{15}N_2$ concentration occurred within 1 to 2 hours (data not shown), as found by Fernandez and Warembourg (1983). Leaks occurred in many of the replicates during the labelling period, which was not surprising due to the complexity of the system (Fig. 3; Sims et al., 1983). Additional $^{15}N_2$ was added when necessary and a time-weighted average ^{15}N concentration was calculated for the 48-hour labelling period.

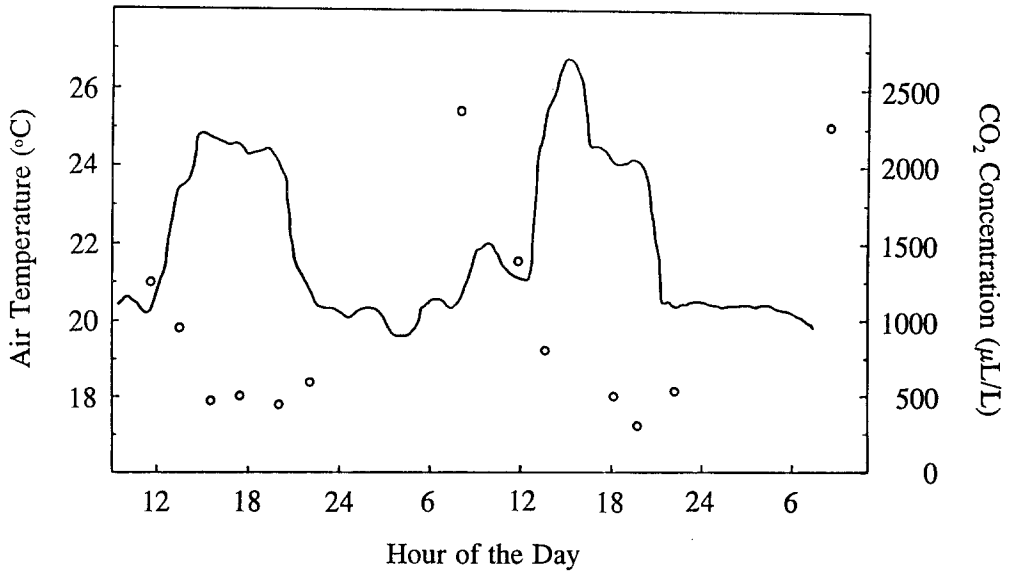

Fig. 2. Time course of air temperatures (solid line) and CO_2 concentrations (open circles) in one replicate group of three drainage lysimeters during a 48-hour labelling period. Air temperatures were logged every 15 minutes and are plotted as a 4-point running mean.

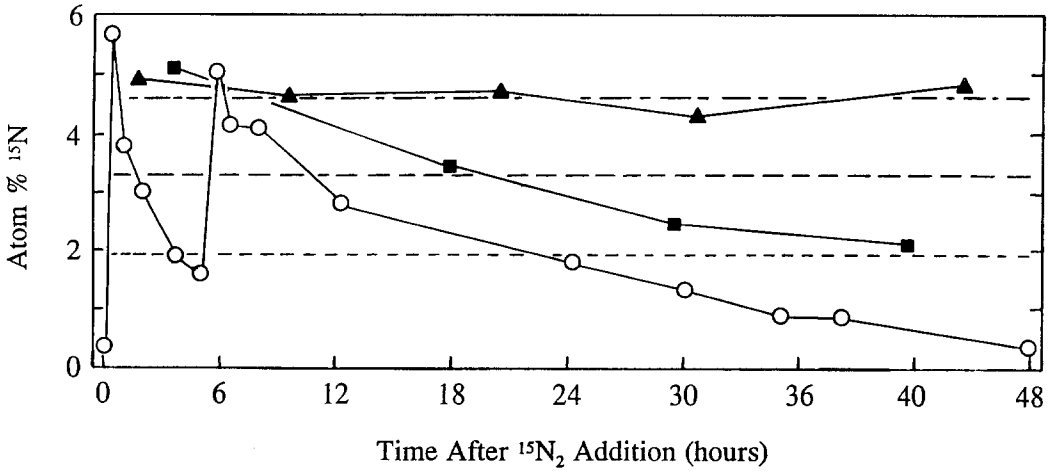

Fig. 3. Examples of ^{15}N concentrations in circulating gas of three replicates during a 48-h labelling period. In one case, extra $^{15}N_2$ was added during the period to increase the label. Time-weighted mean ^{15}N concentrations are shown for each case as broken horizontal lines.

Sampling

After 2 days of labelling, the Saran bags were removed, herbage was harvested, and greenhouse atmosphere was circulated through the columns to purge the $^{15}N_2$ gas. The succeeding replicate was labelled with a new $^{15}N_2$-Ar-O_2 gas mixture for the next 2 days.

After a 2-day 'equilibration' period, six replicate sets of lysimeters were sampled. Each replicate set of lysimeters was removed and cut open longitudinally. Crowns were separated from roots below the lowest visible shoot. Topsoil and subsoil horizons were separated, thick roots (including secondary roots >2 mm diam.) and thin roots (with adhering nodules) were removed from the soil by sieving sequentially through 10-, 5-, and 2-mm screens, followed by hand picking with forceps. Rhizosphere soil was defined as soil adhering to the roots and nodules during this processing; bulk soil was all other soil.

Plants in the other six replicates were allowed to regrow for 35 to 37 days before herbage harvest and root and soil samplings, which were conducted as in the first sampling period (beginning 21 February 1989). Consistent with the first sampling protocol, root sampling was delayed for 2 days after herbage harvest.

Samples from one longitudinal half of each lysimeter were analyzed for composition of soil and roots. Samples from the other half were analyzed for soil solution composition, which will be reported in a subsequent paper. Rhizosphere soil was separated from the roots by 60 second sonication in sodium phosphate buffer, pH 7.0 (Lory et al., 1992). Roots were removed from the solution, rinsed with fresh buffer solution, and, if necessary, rinsed again in deionized water.

All aliquots of buffer were combined for each horizon and dried under heated, acid-scrubbed air on a hot plate at about 60 °C before rhizosphere soil dry mass measurements were made. Rhizosphere dry mass was corrected for content of phosphate buffer salts, calculated from known volumes added during root washing (Lory et al., 1992). Several grams of rhizosphere soil were collected using this method, although it required about 800 hours of labor over 12 consecutive days to remove root and soil samples from 18 lysimeters (six replicates of three treatments). Extreme care was taken during all steps of this labor-intensive process to ensure that soil samples were completely free of visible plant tissue and that cross-contamination among samples did not occur.

Total bulk soil moist mass was determined and subsamples were taken for water content and elemental analysis. Herbage, crown, and root tissues were dried in a forced air oven at 70 °C and finely pulverized in a ball mill.

Chemical analysis

Samples of herbage, crowns, roots, rhizosphere soil, and bulk soil were analyzed for total C, total N, and ^{15}N concentration on a Carlo Erba NA1500 Analyzer (Carlo Erba Strumentazione, Milan, Italy) interfaced to a Tracermass Isotope Mass Spectrometer (Europa Scientific, Cheshire, UK) at the Univ. of Nebraska, Lincoln, Nebraska, USA.

Calculation of N_2 fixation was based on the time-weighted average ^{15}N enrichment of the circulating gas above natural abundance of the atmosphere ($^{15}N_{gas}$) for each replicate and by subtracting the ^{15}N enrichment of the plant or soil fraction of the nonfixing (subscript 'nf') from the fixing (subscript 'f') germplasms:

$$\text{Proportion of N from fixation} = \frac{^{15}N_f - {}^{15}N_{nf}}{^{15}N_{gas}} \quad (1)$$

These proportions were multiplied by the amount of N in the plant or soil fraction to obtain the mass of fixed N in each fraction. Extrapolation of N_2 fixation for the 35-day regrowth period prior to labelling was made by assuming the actual rate averaged 58% of the measured daily rate [based on a weighted mean of 36% of the measured daily rate during the first 23 days after harvest and 100% of the measured rate for the next 12 days (Vance et al., 1979)].

Small amounts of soil could be seen on the cleaned root surfaces under magnification and some samples contained sufficient entrained soil (principally sand) to alter the total C analysis. We calculated total soil contamination of root samples by:

$$\text{mass}_c(g) = \frac{[446 - C_i(\text{mg g}^{-1})] \times \text{mass}_i(g)}{[446 - C_s(\text{mg g}^{-1})]} \quad (2)$$

where 446 is the assumed total C concentration (mg g^{-1}) of noncontaminated root tissue (determined from a large number of samples and Breland and Bakken, 1991) and 'c', 'i', and 's' refer to contaminant, initial sample, and rhizosphere soil, respectively. Root tissue N concentration was corrected by:

$$N_r(\text{mg g}^{-1}) = \frac{[N_i(\text{mg g}^{-1}) \times \text{mass}_i(g)] - [N_s(\text{mg g}^{-1}) \times \text{mass}_c(g)]}{[\text{mass}_i(g) - \text{mass}_c(g)]} \quad (3)$$

where 'N' was total N concentration and subscript 'r' refers to the corrected root tissue. The assumption that entrained soil in the root mass has C and N concentrations equal to clean rhizosphere soil is conservative; sand grains likely would have much smaller concentrations of both elements. Nitrogen content of contaminant soil was always less than 0.5% (wt/wt) that of the thin root N content. We have used this technique to correct fine root and nodule N concentrations in other work (Dubach and Russelle, 1992). Examples of results from these calculations are shown in Table 1.

Statistical analysis

Dry mass, N concentration, and N content of the various plant and soil fractions were subjected to analysis of variance, with single degree of freedom comparisons between germplasms (Ineffective Saranac vs. effective symbioses; Saranac vs. 'excreting'), using 'contrast' statements in SAS (1987). The design was a full factorial arrangement of treatments (germplasm and sampling time) in a randomized complete block with six replicates. Calculated fixed N contents of the various plant and soil fractions were compared by paired t-test between Saranac and the 'excreting' line. All statistical comparisons were made using SAS (1987).

Results and discussion

Plant dry mass

Plants and effective nodules produced markedly more dry mass than Ineffective Saranac (Table 2). There were no significant differences in growth between Saranac and the 'excreting' line at the first sampling, but by the second sampling time the 'excreting' line produced less herbage and had smaller masses of both thick and thin roots in the subsoil than Saranac. Herbage regrowth comprised a smaller proportion of total phytomass at the second sampling; dry mass in crowns and secondarily thickened roots continued to increase. Herbage production of Ineffective Saranac was very limited at the second sampling. The combination of soil N and supplemental fertilizer N were evidently inadequate to sustain rapid growth of this germplasm.

Tap roots were trimmed when plants were transplanted in this experiment and the mass of thick roots reported here may be lower than would be expected for directly seeded plants.

Table 1. Examples of corrections made to thin root N concentrations after calculating adhering rhizosphere soil mass, assuming root C concentration should have been 446 g kg^{-1} and contaminant soil N concentration was equal to that of the rhizosphere soil (see Equations 1 and 2). The highest rhizosphere N concentration was from topsoil, other examples were from subsoil

Measured thin root [C] (g kg^{-1})	Thin root dry mass (g)	Calculated contaminant soil mass (g)	Rhizosphere soil [N] (g kg^{-1})	Thin root [N]	
				Measured (g kg^{-1})	Corrected (g kg^{-1})
323	2.04	0.303	0.383	19.7	27.1
324	3.43	0.493	0.517	20.8	28.5
342	3.02	0.399	0.584	21.4	27.8
398	3.84	0.108	0.542	25.0	28.0
416	4.86	0.199	1.665	27.7	29.7

Table 2. Dry mass (g/plant) of alfalfa tissue and adhering rhizosphere soil at the first sampling (2 to 4 days) and second sampling (35 to 37 days) after ^{15}N labelling

Component	First sampling			Second sampling		
	Saranac	'Excreting'	Ineffective Saranac	Saranac	'Excreting'	Ineffective Saranac
Herbage	2.4 a[x]	2.4 a	1.0 b	2.5 a	1.9 b	0.2 c
Crown	2.5 a	2.6 a	1.8 b	3.6 a	3.0 a	1.5 b
Thick roots[z]	1.7 a	1.6 a	1.1 b	3.9 a	2.4 b	1.4 c
Thin roots[y]						
Topsoil	1.2 a	1.1 a	0.7 b	2.0 a	1.6 a	1.0 b
Subsoil	0.8 a	0.7 a	0.5 b	1.5 a	0.8 b	0.4 c
Rhizosphere soil						
Topsoil	12.1 a	9.3 ab	8.1 b	12.0 a	8.8 b	4.3 c
Subsoil	14.6 a	11.7 a	14.9 a	12.9 ab	15.8 a	7.3 b

[z] Includes any attached nodules

[y] Includes any attached nodules and all unattached nodules

[x] Values followed by the same letter within a row and sampling time are not significantly different ($p > 0.05$) according to single degree of freedom comparisons

However, the general relationships of dry mass among the plant organs we measured are similar to those reported by others (Pettersson et al., 1986).

Plant nitrogen concentration and content

As expected, tissue N concentrations were lower in Ineffective Saranac than in the effective symbioses, whereas few differences were observed between the two effective germplasms (data not shown). Over all germplasms, total N concentrations in herbage ranged from about 30 to 43 g kg^{-1} and total N concentrations in crown tissue were considerably lower, ranging from about 9 to 23 g kg^{-1}. Thickened roots contained from 7 to 25 g N kg^{-1}. As sampled and after washing, thin roots contained from 13 to 30 g kg^{-1}, consistent with other findings (Lory et al., 1992). However, after correction for soil contamination, thin root N concentration increased to between 20 and 34 g kg^{-1}. These latter values appear to be more consistent with tissue that contains relatively little lignin or other secondary cell wall material and which includes nodules.

Accumulation of total N in plant organs varied according to the effectiveness of the symbiosis, with Ineffective Saranac containing less total N than the two other germplasms (Table 3). About one-half of total plant N was contained in the root system of plants with effective symbioses at the final sampling, whereas the ineffective germplasm had about two-thirds of its total N in the roots. Thin roots contained approximately as much N as herbage at the final sampling of the effective symbioses, equivalent to 190 kg N ha^{-1}, assuming a stand density of 225 plants m^{-2}. Total N in the ineffective germplasm was lower in plants sampled after herbage regrowth than in those sampled earlier, whereas N content of effective symbioses remained the same or increased. This decline in plant dry mass and N content of the ineffective germplasm may have resulted from the continued stress of low N supply or some unmeasured effect. Ineffective Saranac can produce herbage yields similar to effective Saranac when N supply is not limiting (Barnes et al., 1992).

Effective Saranac contained more symbiotically fixed N than the 'excreting' line in many organs (Table 4), and in the second sampling these differences followed trends seen in total N content (Table 3). Rates of symbiotic N_2 fixation ranged from 46 to 77 μg plant^{-1} hour^{-1}, assuming that the label was present for a 48-hour period. These values are similar to those for *Trifolium* spp. (Hopmans et al., 1983; Montange et al., 1981) and alfalfa (calculated as approx. 54 μg plant^{-1} hour^{-1} in midsummer; Heichel, 1987). The similarity among these values suggests that the increased CO_2 concentrations inherent in the labelling process (Fig. 2) did not have a significant impact on N_2 fixation rates.

Table 3. Nitrogen content (mg/plant) of alfalfa tissues and adhering rhizosphere soil at the first sampling (2 to 4 days) and second sampling (35 to 37 days) after ^{15}N labelling

Component	First sampling			Second sampling		
	Saranac	'Excreting'	Ineffective Saranac	Saranac	'Excreting'	Ineffective Saranac
Herbage	88 a[w]	82 a	32 b	89 a	82 a	6 b
Crown	48 a	60 a	22 b	60 a	59 a	13 b
Thick roots[z]	41 a	40 a	12 a	81 a	61 a	10 b
Thin roots[y]						
Topsoil	36 a	36 a	17 b	62 a	49 a	21 b
Subsoil	21 a	20 a	10 b	37 a	21 a	6 b
Rhizosphere soil[x]						
Topsoil	7 a	5 ab	3 b	12 a	8 a	3 a
Subsoil	3 a	2 a	1 a	6 a	4 a	2 b

[z] Includes any attached nodules
[y] Includes any attached nodules and all unattached nodules
[x] Increase in N content calculated using rhizosphere soil mass and the difference between N concentrations in the rhizosphere and bulk (nonrhizosphere) soil
[w] Values followed by the same letter within a row and sampling time are not significantly different ($p > 0.05$) according to single degree of freedom comparisons

Table 4. Content of symbiotically fixed N of alfalfa organs and adhering rhizosphere soil and distribution of symbiotically fixed N in the plant at the first sampling (2 to 4 days) and second sampling (35 to 37 days) after ^{15}N labelling

Component	First sampling				Second sampling			
	Sar[z]	'Excr'	Sar	'Excr'	Sar	'Excr'	Sar	'Excr'
	Content ($\mu g\ plant^{-1}$)		Distribution[y] (%)		Content ($\mu g\ plant^{-1}$)		Distribution (%)	
Herbage	640 a[v]	670 a	–	–	1300 a	1100 a	36 a	46 a
Crown	880 a	630 b	47 a	42 a	640 a	650 a	18 a	27 a
Thick roots[x]	570 a	250 b	30 a	17 a	780 a	310 b	21 a	13 a
Thin roots[w]								
Topsoil	340 a	460 a	18 a	30 a	500 a	220 b	14 a	9 a
Subsoil	100 a	170 a	5 a	11 a	420 a	140 b	12 a	6 b
Rhizosphere soil								
Topsoil	24 a	26 a	–	–	39 a	11 b	–	–
Subsoil	14 a	4 a	–	–	13 a	6 a	–	–

[z] Sar = Saranac; 'Excr' = 'excreting' line
[y] Excluding herbage at first sampling time to show redistribution during regrowth
[x] Includes any attached nodules
[w] Includes any attached nodules and all unattached nodules
[v] Values followed by the same letter within a row, measurement type, and sampling time are not significantly different ($p > 0.10$) according to single degree of freedom comparisons

We cannot make direct comparisons of symbiotically fixed N content between sampling times, because of disparities in the total amount of ^{15}N labelling at the two times. These differences probably arose primarily from variability among plants in growth rate and symbiotic N_2 fixation potential, because we sampled relatively few plants in these cross-pollinated populations. To compare sampling times, we assumed that the *relative* distribution of recently fixed N in the remaining plant organs after herbage harvest was similar for the two sampling groups (Table 4). Plants in both sampling groups had experienced similar growth conditions and management, and shoots were removed from all plants 2 days after labelling. Thus, labelled N found in herbage regrowth originated in crowns and roots.

Between 36 and 46% of the fixed N present in

crowns and roots of effective symbioses 2 days after shoot harvest was translocated into herbage regrowth during the next 35 days (Table 4). These values can be compared with those of Volenec et al. (1991), who estimated that up to 50% of soluble root and crown N is utilized in shoot regrowth. Ta et al. (1990) concluded that about 25% of total root N was translocated into regrowing shoots after harvest of 8-week-old alfalfa plants. In young, uninoculated alfalfa, 80% of the N in unharvested leaves on the crown, 68% of tap root N, and 52% of lateral root N was translocated to new stems and leaves during regrowth (Kim et al., 1991). Studies with subterranean clover (*Trifolium subterranean* L.) over three regrowth periods showed that between 100 and 143% of the N initially present in crowns and roots was used for shoot regrowth (Phillips et al., 1983). Relative amounts of N available for translocation likely depend on the age of the organs, because N in structural proteins generally increases with secondary growth. Based on charges in the relative distribution of fixed N in our experiment, most of the retranslocated N apparently came from the crown and thick roots in Saranac, whereas it came from crowns and thin roots in the 'excreting' line (Table 4).

Rhizosphere soil mass and nitrogen

Rhizosphere soil mass varied among the plant germplasms, but did not exceed 27 g plant^{-1} (Table 2). We observed that more rhizosphere soil was obtained from the distal ends of roots than from other portions. Rhizosphere soil recovery also was greater at intermediate soil water contents. We were unable to recover all roots from the soil, but estimate that at least 95% of the total root length was removed. This undersampling of total rhizosphere soil probably resulted in underestimates of net N loss to the rhizosphere, but the extent of undersampling is unknown.

In the topsoil, total N concentration in rhizosphere soil ranged from 1.2 to 1.7 g kg^{-1} and N concentration in bulk soil averaged 0.8 g N kg^{-1}; in subsoil, total N concentration ranged from 0.5 to 0.7 g kg^{-1} in the rhizosphere and averaged 0.3 g kg^{-1} in the bulk soil. Differences in rhizosphere soil N concentration due to alfalfa germplasm were significant only in the topsoil at the first sampling (data not shown). The increase in total N in rhizosphere soil has been noted elsewhere (Bartholomew and Clark, 1950; Breland and Bakken, 1991; Lory et al., 1992) and may be due to immobilization of N by abundant microbial populations surrounding the root. Total N accretion in the rhizosphere was equivalent to between 7.5 and 11.5 g N kg^{-1} thin root C present at both sampling times, congruent with estimates of 9 g N kg^{-1} root C for 96-day-old white clover (*Trifolium repens* L.; Breland and Bakken 1991).

An increase in N content of rhizosphere soil over bulk soil was observed for all germplasms, with greater increases in the effective symbioses (Table 3). Larger accumulation of N in the rhizosphere of effective symbioses was due both to increased amount of rhizosphere soil from the greater thin root mass, as well as higher concentrations of N in the rhizosphere soil. We speculate that the larger, N_2-fixing germplasms exuded more C, which would support larger microorganism populations (Breland and Bakken, 1991), and used more water, which would transport more inorganic N from the bulk soil to these rhizosphere microorganisms.

Total net amounts of rhizosphere N originally derived from symbiotic N_2 fixation ranged from 17 to 52 μg plant^{-1} (Table 4). Assuming that all fixation of labelled N_2 gas occurred in 2 days, using plant populations of 225 m^{-2}, and extrapolating the values to an entire 35-day regrowth period, the observed net deposition of symbiotically fixed N was equivalent to no more than 1.2 kg ha^{-1}. The net amount of symbiotically fixed N found in the rhizosphere represented 1.5% or less of the total fixed N in the plants. This value is similar to that obtained in another greenhouse study (Lory et al., 1992), in which the same technique of separating rhizosphere soil from bulk soil was employed, but which relied on long-term indirect ^{15}N labelling techniques. Our results demonstrate that the 'excreting' line probably transfers N to neighboring plants via a mechanism other than net loss of fixed N from living roots. Hence, the term 'excreting' may be a misnomer.

Our results contrast with those found in soy-

bean (Ofosu-Budu et al., 1990). These researchers found that about 30% of the N fixed between flowering and pod-filling stage was excreted. However, they recognized that their methodology of bathing the soybean roots in flowing, N-free nutrient solution both maximized losses of compounds from the roots and minimized possible reabsorption. Ruschel et al. (1979) reported apparent losses of 17% of total symbiotically fixed N from young *Phaseolus vulgaris* L. and from soybean grown in soil-vermiculite mixtures, but they exposed their plants to a temporary vacuum before and after exposure to ^{15}N-labelled gas. Other reports of losses of symbiotically fixed N from alfalfa roots and nodules have been about one order of magnitude smaller than from *Phaseolus* or *Glycine*, even when exposed to nutrient solutions of low osmotic potential (Brophy and Heichel, 1989; Ta et al., 1986).

In our experiment, symbiotically fixed N could have moved away from the rhizosphere and into the bulk soil or could have been reabsorbed by the plant. We do not expect that these processes were significant (Lory et al., 1992). Losses of N through leaching were insignificant, once the transplant had become established (data not shown). The congruence of our results, obtained in nonsterile soil, with those in hydroponics (Ta et al., 1986) and in sterile sand (Brophy and Heichel, 1989) lends support to our conclusion that losses of symbiotically fixed N from living roots and nodules of alfalfa are too small to explain reported rates of either N accretion in soil during alfalfa growth or N transfer to associated nonlegumes cited earlier. Net N accumulation in soil during alfalfa growth is likely due to other processes, such as decomposition of roots, nodules, and above ground litter.

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